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Abstract

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Disciplines

Chemistry

Comments

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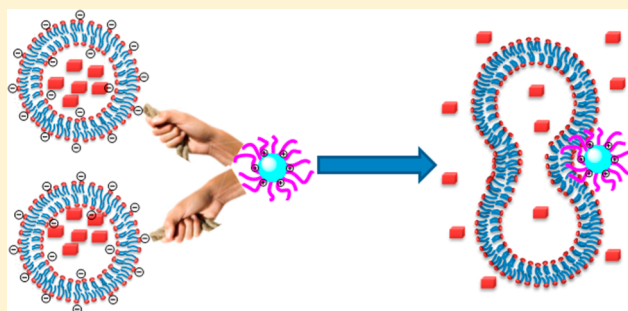
Tunable Fusion and Aggregation of Liposomes Triggered by Multifunctional Surface-Cross-Linked Micelles

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S Supporting Information

ABSTRACT: Water-soluble organic nanoparticles were prepared by cross-linking the micelles of a tripropargylated cationic surfactant by a diazide cross-linker in the presence of Cu(I) catalysts. The nanoparticles were decorated with hydrophilic ligands of different lengths on the surface. By interacting with negatively charged liposomes through tunable electrostatic interactions, these nanoparticles induced fusion and leakage of large unilamellar vesicles (LUVs). Fusion or aggregation of the membranes was highly sensitive to the rigidity and phase structures of the membranes, enabling thermally gated fusion to occur within a very narrow window of temperature change.



Membrane fusion is a key step in many biological processes including fertilization, cell infection by enveloped viruses, and intracellular molecular trafficking.¹ For two lipid membranes to fuse, they have to overcome significant steric/electrostatic repulsion before merging with each other. Opening of aqueous fusion pores allows the two vesicles to connect their aqueous contents. Biological fusion is tightly regulated by specific fusion proteins.^{1,2} Developing a detailed understanding of the fusion mechanism is of great fundamental and practical significance. Fusion inhibitors, for example, could become useful antiviral agents, and materials mimicking enveloped viruses in cell entry may be highly useful for the intracellular delivery of pharmaceuticals.

Synthetic fusogens are simplified small- or macromolecular systems capable of inducing membrane fusion in a controllable manner.³ In addition to providing mechanistic insight into the fusion process, their ease of synthesis makes them promising candidates as drug delivery vehicles. Chemists have employed metal–ligand complexation,⁴ reversible boronate ester bonds,^{5,6} and DNA base-pairing^{7,8} to overcome repulsive forces of membranes. Some researchers used bioinspired peptides for similar purposes.^{9–13} Bong and co-workers in recent years reported a number of strategies to control fusion. Some of the interactions (e.g., vancomycin/D-Ala-D-Ala)^{14,15} in their fusogens were adapted from biology and others (e.g., cyanuric acid–melamine)^{16,17} were purely synthetic. The latter example was particularly intriguing because hydrogen-bonded systems are normally assumed to be ineffective for water-based applications.

Herein, we report easy-to-synthesize, water-soluble surface-cross-linked micelles (SCMs) that induce membrane fusion through tunable electrostatic interactions. The size of the SCM was critical to its activity. The electrostatically induced fusion was highly sensitive to the phase structure of the lipids,

enabling thermally gated fusion within a very narrow window of temperature change.

The synthesis of the SCMs was shown in Scheme 1. Briefly, 4-dodecyloxybenzyltripropargyl ammonium bromide (**1**) forms micelles with a dense layer of alkyne groups on the surface. The addition of 1 equiv of water-soluble cross-linker **2** and Cu(I) fixes the dynamic assemblies of surfactants into water-soluble nanoparticles with numerous residual alkyne groups on the surface. According to our previous study,¹⁸ the majority of the surfactant molecules under such reaction conditions underwent two cycloaddition reactions and some reacted three times—fully in line with the high efficiency of the click reaction. Post-functionalization of the alkynyl-SCM was achieved readily by another round of click chemistry using various azide-functionalized compounds **3–5** in a one-pot reaction.

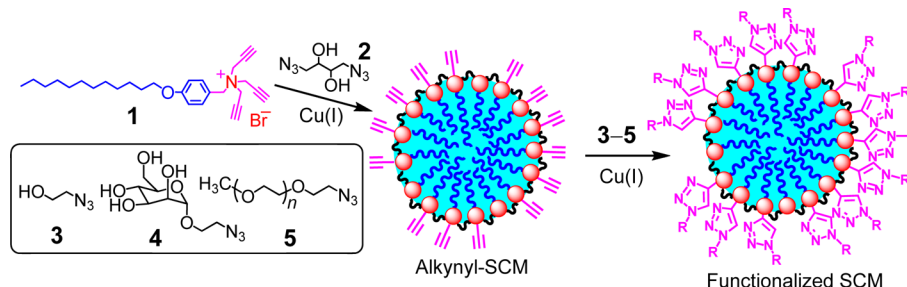
The hydrodynamic diameters of the hydroxyl-, mannose-, and PEG-functionalized SCMs were determined by DLS to be ca. 14, 20, and 150 nm, respectively (Figures 1–3S, Supporting Information). Similar materials were characterized by TEM previously and found to be spherical in shape.¹⁸ To understand how these differently-sized particles interact with liposomes, we prepared large unimolecular vesicles (LUVs) containing carboxyfluorescein (CF) by the membrane extrusion method. The liposomes, ca. 120 nm after extrusion, were made of neutral POPC and 10 mol % anionic POPG. This formulation is frequently used in liposomal research and mimics the composition of bacterial membranes. The negative charges of the liposomes make them colloiddally more stable. Moreover, the charges allow the liposomes to interact with the positively charged SCMs through Coulombic interactions.

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Scheme 1. Synthesis of Functionalized Surface-Cross-Linked Micelles (SCMs)



CF is a water-soluble fluorescent dye that displays self-quenching at high concentrations (>50 mM).¹⁹ If the membranes are unaffected by SCMs, CF would stay within the liposomes and emit weakly due to self-quenching. If the SCMs cause any leakage of the liposomes, CF would escape, get diluted, and display enhanced emission. The percent leakage is generally calculated based on the complete release at the end of the assay triggered by a nonionic surfactant, 1% Triton X-100.

The leakage assay showed that all three SCMs were able to trigger the release of CF (Figure 1). SCM-OH (i.e., SCM

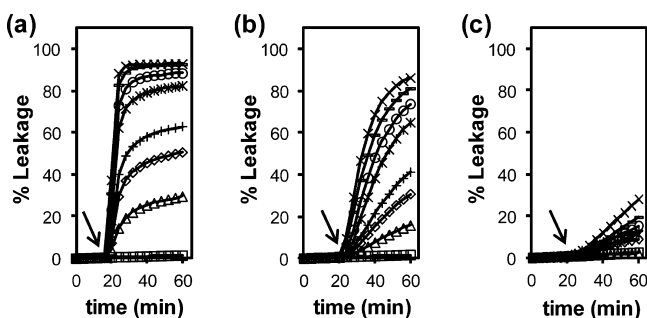


Figure 1. Percent CF leakage from POPC/POPG LUVs upon the addition of different concentrations of (a) SCM-OH, (b) SCM-mannose, and (c) SCM-PEG at 25 °C. The concentrations of the (cross-linked) surfactant in the SCMs were 0, 0.1, 0.2, 0.3, 0.5, 0.7, 1.0, and 1.5 μ M from bottom to top. [phospholipids] = 2.9 μ M. The concentration of CF was 50 mM within the LUVs. The arrow indicates the addition of SCM at 20 min.

functionalized by 2-azidoethanol 3) was the most effective among all, triggering significant leakage at 0.1 μ M of the (cross-linked) surfactant. Note that the CF leakage was nearly instantaneous at the addition of SCM-OH and exceeded 90% when the concentration of the cross-linked micelles reached 1.0 μ M.

Surface-functionalization overall reduced the potency of the SCMs. As the size of the surface groups increased from $\text{CH}_2\text{CH}_2\text{OH}$ to mannose to PEG, CF leakage became noticeably slower (Figure 1b,c). For the PEGylated SCM, the leakage was less than 30% after 60 min even at the highest tested concentration.

Tunable electrostatic interactions between the SCMs and the liposomes were confirmed by zeta-potential measurements. As seen in Figure 4S, the POPC/POPG LUVs had a zeta-potential of -5.8 mV in the HEPES buffer, apparently due to the negatively charged POPG lipids. The SCMs were all positively charged, as expected. The zeta-potentials decreased with increasing length of the surface ligands, being 37.3, 32.3, and

18.0 for SCM-OH, SCM-mannose, and SCM-PEG, respectively.

A combination of lipid-mixing assay and DLS study shed light on the leakage mechanism. In the lipid-mixing assay, one batch of LUVs containing 1 mol % NBD- and rhodamine-functionalized lipids was mixed with another batch of unlabeled LUVs. If any process exists that causes membrane disintegration or fusion, the labels would be diluted, lowering the fluorescence resonance energy transfer (FRET) from NBD to rhodamine.²⁰ Indeed, significant fusion of the POPC/POPG membranes was induced by SCM-OH. The percent fusion of the membranes was over 60% when the concentration of the (cross-linked) surfactant in the SCM was 1.5 μ M (Figure 2a). Because the percent CF leakage was about 90% at the same concentration of SCM (Figure 1a), membrane fusion was responsible for most, if not all, of the leakage.

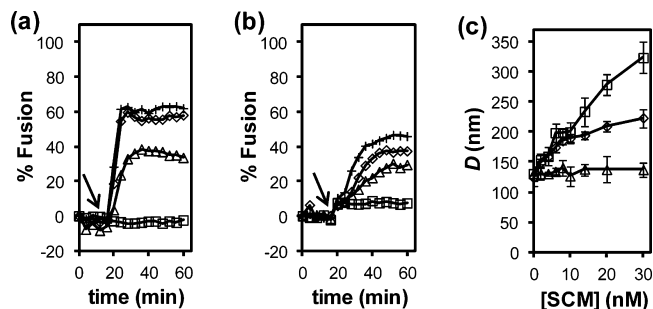


Figure 2. (a) Percent fusion of POPC/POPG LUVs triggered by (a) SCM-OH and (b) SCM-mannose at 25 °C. The concentrations of the (cross-linked) surfactant in the SCM were 0, 0.5, 1.0, and 1.5 μ M from bottom to top. The arrow indicates the addition of SCM at 20 min. (c) DLS diameter of the liposomes after the addition of SCM-OH (\square), SCM-mannose (\diamond), and SCM-PEG (Δ). [phospholipids] = 2.9 μ M.

The mannose-functionalized SCM seemed to trigger the CF efflux by the same mechanism. At 1.5 μ M of the (cross-linked) surfactant, the percent fusion was nearly 50% (Figure 2b), comparing favorably with the 85% CF leakage under the same condition (Figure 1b). Note that the CF leakage and membrane fusion had similar profiles. SCM-OH, in general, induced abrupt changes, whereas SCM-mannose was more gradual (compare the curves with >0.5 μ M SCM in Figure 1a and b with those in Figure 2a and b). Bergstrand and co-workers found that liposome leakage sometimes proceeded more rapidly than lipid mixing, suggesting that liposomes could leak prior to aggregation and lipid rearrangement.²¹ In our case, when the leakage and lipid mixing were normalized, it was clear that the two processes occurred simultaneously (Figure 5S). The nearly identical time responses observed in two different

assays strongly support that membrane fusion was responsible for the CF leakage.

Membrane fusion was also confirmed by DLS. As shown in Figure 2c, both SCM-OH (\square) and SCM-mannose (\diamond) increased the size of the liposomes significantly, following a nearly linear relationship to the SCM concentration. Once again, the size increase was most significant with SCM-OH. It should be noted that no sedimentation or precipitation of liposomes was observed in our experiments.

SCM-PEG, on the other hand, behaved differently in every aspect. As shown earlier, it was quite ineffective at inducing CF leakage. The lipid-mixing assay showed negligible fusion (Figure 6S). DLS, in the meantime, revealed that the liposomes stayed unchanged in size upon the addition of the PEGylated SCMs (Figure 2c, Δ).

The above results may be understood from the tunable Coulombic interactions between the liposomes and the SCMs. Because the charges of an SCM come from the ammonium headgroups of the cross-linked surfactants, their locations on the SCM are fixed. Surface-functionalization adds nonionic hydrophilic ligands to the surface of the SCM nanoparticle. These ligands essentially represent an “insulating” layer when the SCMs adsorb unto the oppositely charged liposomes. A thicker insulating layer weakens the Coulombic interactions, making the larger SCMs less capable of inducing CF leakage and membrane fusion. Because SCM-PEG caused neither fusion nor size change in the liposomes, the small extent of leakage in Figure 1c most likely comes from local destabilization of the membranes.

The smaller SCMs (SCM-OH and SCM-mannose) thus were able to overcome the repulsion forces between negatively charged liposomes quite effectively. We hypothesized that, even when liposomes were brought together by the SCMs, whether membrane fusion took place would still depend on other factors such as the rigidity of membranes. Membranes that deform easily should fuse more easily than those that are rigid.

To test the hypothesis, we studied the effects of the SCMs on DPPC/DPPG liposomes. The LUVs had a zeta-potential of -13.4 mV at 25°C in the HEPES buffer (Figure 4S). The saturated lipids have a gel–liquid-crystalline transition temperature of 41°C . The membranes, thus, should be quite rigid at room temperature. Indeed, no CF efflux was observed with any of the three SCMs at 25°C (see Figure 3a for SCM-OH and

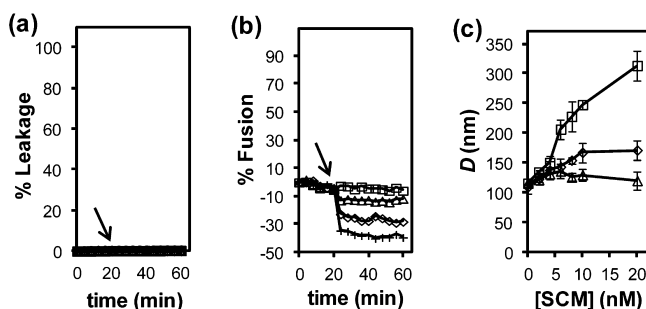


Figure 3. (a) Percent CF leakage from DPPC/DPPG LUVs upon the addition of different concentrations of SCM-OH at 25°C . (b) Percent fusion of DPPC/DPPG LUVs triggered by SCM-OH at 25°C . The concentrations of the (cross-linked) surfactant in the SCM were 0, 0.5, 1.0, and $1.5\ \mu\text{M}$ from bottom to top. The arrow indicates the addition of SCM at 20 min. (c) DLS diameter of the DPPC/DPPG liposomes after the addition of SCM-OH (\square), SCM-mannose (\diamond), and SCM-PEG (Δ) at 25°C .

Figure 7S for SCM-PEG). Interestingly, the lipid-mixing assay gave negative numbers for the membrane fusion (Figure 3b). In other words, instead of decreasing the FRET from NBD to rhodamine, the addition of the SCM-OH enhanced the energy transfer. This behavior normally takes place when liposomes aggregate without the membranes fusing together.²² Under such a situation, the energy transfer within a labeled liposome remains effective and additional energy transfer occurs when multiple labeled liposomes come close—the Förster radius of the two fluorophores is ~ 5 nm.²⁰ Our DLS study confirmed the conclusion, showing a quick increase of the liposome size upon the addition of SCM-OH (Figure 3c, \square). As expected from the electrostatic model, aggregation was less prominent with SCM-mannose (\diamond) and absent with SCM-PEG (Δ).

The above experiments demonstrated that rigid membranes are quite resistant to the electrostatically induced membrane fusion. The results are reasonable because fusion requires substantial reorganization of the membranes, which should be more difficult when lipid molecules have low mobility. The situation changed as soon as the membranes went into the liquid-crystalline state. As shown by Figure 4a, the nano-

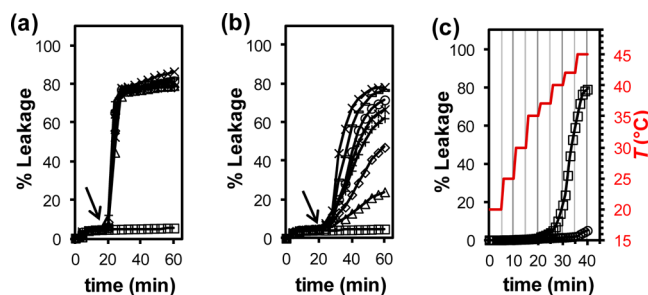


Figure 4. Percent leakage of CF from DPPC/DPPG LUVs upon the addition of different concentrations of (a) SCM-OH and (b) SCM-PEG at 42°C . The concentrations of the (cross-linked) surfactant in the SCM were 0, 0.1, 0.2, 0.3, 0.5, 0.7, 1.0, and $1.5\ \mu\text{M}$ from bottom to top. The arrow indicates the addition of SCM at 20 min. The concentration of CF was 50 mM within the LUVs. (c) Percent CF leakage from DPPC/DPPG LUVs in the presence (\square) and absence (\diamond) of $1.0\ \mu\text{M}$ SCM-OH during temperature change. [phospholipids] = $2.9\ \mu\text{M}$.

particles became extremely potent. Most remarkably, even $0.1\ \mu\text{M}$ of the (cross-linked) surfactant in SCM-OH was able to leak over 80% of the CF from the DPPC/DPPG LUVs at 42°C , whereas the same could only be achieved at much higher levels of the SCMs for POPC/POPG LUVs (Figure 1a). Similar observations were made for the SCM-mannose (Figure 8S).

The dependence of leakage on the lipid phase was demonstrated most clearly by a temperature-controlled experiment, in which the sample was heated from 20 to 45°C with step-changes every 5 min (Figure 4c, red). In the absence of the SCM, little CF efflux occurred from the DPPC/DPPG liposomes. With as little as $0.1\ \mu\text{M}$ of the cross-linked surfactant in SCM-OH, the situation was enormously different. Almost no leakage occurred before 40°C and the majority of all the leakage took place between 40 and 42°C . Apparently, the aggregated²³ liposomes were able to resist the electrostatic stress right until the phase transition occurred.

The above results indicate that the saturated DPPC/DPPG membrane just above the phase-transition temperature was *far more sensitive* to the electrostatic stress than the monounsaturated

rated POPC/POPG. SCM-PEG showed the same trend. Low activity was observed with the PEGylated SCMs for POPC/POPG liposomes (Figure 1c). Although not as effective as SCM-OH and SCM-mannose, SCM-PEG induced significant leakage of CF at 42 °C for the saturated liposomes, most likely due to local destabilization of the membranes. Nearly 80% of CF was released by 1.5 μ M of the (cross-linked) surfactant in the PEGylated SCM (Figure 4b).

The phase-transition temperature of POPC/POPG is -2 °C. At 25 °C, the membrane should be much more fluid-like than DPPC/DPPG at 42 °C right above the latter's phase-transition temperature. Permeability of membranes is known to increase at the phase-transition temperature due to coexisting gel and liquid-crystalline phases.^{24–27} The higher permeability is considered to result from packing defects at the boundaries of solid-lipid phase boundaries. It should be emphasized that, under our experimental conditions, very little CF leakage occurred from DPPC/DPPG liposomes in the absence of the SCMs (Figure 4c, \diamond). Thus, the temperature-triggered release in our case was caused by the vulnerability of the membranes with coexisting domains to the electrostatic stress. A separate study of ours demonstrated that, once the charge of the liposomes was reversed (to positive), membrane fusion disappeared completely.²⁸

Quite likely, mobile lipids in the fluid phase can adjust easily to accommodate both the liposome-SCM electrostatic interactions and the hydrophobic interactions of the lipid tails. Near the phase-transition temperature, on the other hand, lipid rearrangement could only occur within the more mobile domains of liquid-crystalline phase, making it more difficult to relieve the electrostatic stress induced by the SCMs and affording higher leakage as a result.

The most attractive feature of the SCMs is their ease of synthesis and post-functionalization. Ligand attachment can accurately tune the thickness of the insulating layer on the SCM and, in turn, modulate the Coulombic interactions between the nanoparticles and the oppositely charged liposomes. Depending on the rigidity and phase structures of the membranes, well-regulated aggregation and fusion of membranes may result.

■ ASSOCIATED CONTENT

Supporting Information

Liposome preparation, liposome assays, and additional figures. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

- (1) Jahn, R.; Lang, T.; and Südhof, T. C. (2003) Membrane fusion. *Cell* 112, 519–533.
- (2) Harrison, S. C. (2008) Viral membrane fusion. *Nat. Struct. Mol. Biol.* 15, 690–698.
- (3) Marsden, H. R., Tomatsu, I., and Kros, A. (2011) Model systems for membrane fusion. *Chem. Soc. Rev.* 40, 1572–1585.
- (4) Richard, A., Marchi-Artzner, V., Lalloz, M.-N., Brienne, M.-J., Artzner, F., Gulik-Krzywicki, T., Guedeau-Boudeville, M.-A., and Lehn, J.-M. (2004) Fusogenic supramolecular vesicle systems induced by metal ion binding to amphiphilic ligands. *Proc. Natl. Acad. Sci. U. S. A.* 101, 15279–15284.
- (5) Kashiwada, A., Tsuboi, M., and Matsuda, K. (2009) Target-selective vesicle fusion induced by molecular recognition on lipid bilayers. *Chem. Commun.*, 695–697.
- (6) Kashiwada, A., Tsuboi, M., Mizuno, T., Nagasaki, T., and Matsuda, K. (2009) Target-selective vesicle fusion system with pH-selectivity and responsiveness. *Soft Matter* 5, 4719–4725.
- (7) Stengel, G., Zahn, R., and Hook, F. (2007) DNA-induced programmable fusion of phospholipid vesicles. *J. Am. Chem. Soc.* 129, 9584–9585.
- (8) Chan, Y.-H. M., van Lengerich, B., and Boxer, S. G. (2009) Effects of linker sequences on vesicle fusion mediated by lipid-anchored DNA oligonucleotides. *Proc. Natl. Acad. Sci. U. S. A.* 106, 979–984.
- (9) Parente, R. A., Nir, S., and Szoka, F. C. (1988) pH-Dependent fusion of phosphatidylcholine small vesicles - Induction by a synthetic amphipathic peptide. *J. Biol. Chem.* 263, 4724–4730.
- (10) Puyal, C., Maurin, L., Miquel, G., Bienvenüe, A., and Philippot, J. (1994) Design of a short membrane-destabilizing peptide covalently bound to liposomes. *Biochim. Biophys. Acta, Biomembr.* 1195, 259–266.
- (11) Lorin, A., Charlotiaux, B., Fridmann-Sirkis, Y., Thomas, A., Shai, Y., and Brasseur, R. (2007) Mode of membrane interaction and fusogenic properties of a de novo transmembrane model peptide depend on the length of the hydrophobic core. *J. Biol. Chem.* 282, 18388–18396.
- (12) Kashiwada, A., Matsuda, K., Mizuno, T., and Tanaka, T. (2008) Construction of a pH-responsive artificial membrane fusion system by using designed coiled-coil polypeptides. *Chem.—Eur. J.* 14, 7343–7350.
- (13) Marsden, H. R., Elbers, N. A., Bomans, P. H. H., Sommerdijk, N. A. J. M., and Kros, A. (2009) A reduced SNARE model for membrane fusion. *Angew. Chem., Int. Ed.* 48, 2330–2333.
- (14) Gong, Y., Luo, Y., and Bong, D. (2006) Membrane activation: selective vesicle fusion via small molecule recognition. *J. Am. Chem. Soc.* 128, 14430–14431.
- (15) Gong, Y., Ma, M., Luo, Y., and Bong, D. (2008) Functional determinants of a synthetic vesicle fusion system. *J. Am. Chem. Soc.* 130, 6196–6205.
- (16) Ma, M., Paredes, A., and Bong, D. (2008) Intra- and intermembrane pairwise molecular recognition between synthetic hydrogen-bonding phospholipids. *J. Am. Chem. Soc.* 130, 14456–14458.
- (17) Ma, M., Gong, Y., and Bong, D. (2009) Lipid membrane adhesion and fusion driven by designed, minimally multivalent hydrogen-bonding lipids. *J. Am. Chem. Soc.* 131, 16919–16926.
- (18) Zhang, S., and Zhao, Y. (2010) Facile synthesis of multivalent water-soluble organic nanoparticles via “surface clicking” of alkynylated surfactant micelles. *Macromolecules* 43, 4020–4022.
- (19) New, R. R. C. (1990) *Liposomes: A Practical Approach*, IRL Press, Oxford.
- (20) Struck, D. K., Hoekstra, D., and Pagano, R. E. (1981) Use of resonance energy-transfer to monitor membrane-fusion. *Biochemistry* 20, 4093–4099.
- (21) Bergstrand, N., Arfvidsson, M. C., Kim, J.-M., Thompson, D. H., and Edwards, K. (2003) Interactions between pH-sensitive liposomes and model membranes. *Biophys. Chem.* 104, 361–379.
- (22) Rafikova, E. R., Melikov, K., Ramos, C., Dye, L., and Chernomordik, L. V. (2009) Transmembrane protein-free membranes fuse into xenopus nuclear envelope and promote assembly of functional pores. *J. Biol. Chem.* 284, 29847–29859.
- (23) According to Figures 3b and 4c.
- (24) Papahadjopoulos, D., Jacobson, K., Nir, S., and Isac, T. (1973) Phase transitions in phospholipid vesicles. Fluorescence polarization

and permeability measurements concerning the effect of temperature and cholesterol. *Biochim. Biophys. Acta* 311, 330–48.

(25) Blok, M. C., van Deenen, L. L., and De Gier, J. (1976) Effect of the gel to liquid crystalline phase transition on the osmotic behaviour of phosphatidylcholine liposomes. *Biochim. Biophys. Acta* 433, 1–12.

(26) Clerc, S. G., and Thompson, T. E. (1995) Permeability of dimyristoyl phosphatidylcholine/dipalmitoyl phosphatidylcholine bilayer membranes with coexisting gel and liquid-crystalline phases. *Biophys. J.* 68, 2333–41.

(27) Mills, J. K., and Needham, D. (2005) Lysolipid incorporation in dipalmitoylphosphatidylcholine bilayer membranes enhances the ion permeability and drug release rates at the membrane phase transition. *Biochim. Biophys. Acta, Biomembr.* 1716, 77–96.

(28) Li, X., and Zhao, Y. (2012) Protection/deprotection of surface activity and its applications in the controlled release of liposomal contents. *Langmuir* 28, 4152–4159.